An Electron Spin Resonance Study of the Steroid Conformation —6-Oxohelvolic Acid (Triketone)—

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The triketone (6-oxohelvolic acid (IIa) or its tetrahydro-derivative (IIb)) was readily oxidized in air into the anhydride (IIIa or b) in an alkaline solution; it spontaneously turned red or deep green and then gradually lost its color. In this reaction process, the presence of a relatively stable intermediate radical was confirmed by the ESR measurement. As a result of an analysis of the well-resolved proton hyperfine structure of this radical, it was explained that the structure of helvolic acid is of the protostane type: that is, it is a distinctive structure of the *trans*-fusion of the A/B ring juncture and a boat form of the B-ring. A reasonable oxidation mechanism including this intermediate radical was presented.

In general, the physical methods of ORD or CD, NMR, and X-ray diffraction are widely used in conformational analyses of steroids; much valuable information has been thus obtained, as is illustrated in the textbook.[1]

However, although the most exact structural information can be derived from an X-ray analysis, provided that the steroid is obtained in a single crystal form, such an analysis can not tell us about delicate conformational changes in steroids in various solvents. Such information is, though, important for the study of stereochemical reaction processes.

On the other hand, ORD or CD is especially useful for the elucidation of the stereoconformations of steroids or terpenes in solutions. However, even in these cases, the determination has to be done in comparison with compounds of a close structure or of a known absolute configuration.

Apart from these methods, the proton NMR spectroscopy is a powerful method for use in the stereochemistry of steroids, and recent advances in this field, for example, the double-resonance method and high-field resonance method (100 MHz or higher frequency), provide valuable information on the absolute configurations of steroids.

The application of the ESR method to the study of the conformations of macromolecules is rather limited in the field of the stereochemistry of steroids because of necessity for an unpaired electron spin in the molecules. The "spin-labeling" method developed by McConnell *et al.*,[2] which involves attaching a stable simple radical (generally an *N*-oxide radical) on a macromolecule and detecting the subsequent delicate environmental conformational change around the labeled spin, has been proved to be a powerful tool for the study of biomolecules,[2] such as haemoglobin, DNA, and cell membranes. From a slightly different point of view, Russell *et al.*[3] have studied the conformations of various steroids, changing them into "semidione" radicals and treating them with *t*-BuOK in air; they have thus been able to classify the reactivities of $-CH_2$ groups neighboring a carbonyl or hydroxyl group. This method also informs us about the dynamic characters of ring inversion under various environmental conditions.

We are interested in the application of ESR to the stereoconformational studies of steroids, by making use of the extra-high sensitivity (10^{11} spins/gauss) of the ESR method and sensitive changes in hf splittings depending on the slight conformational change in the spin environment.

Helvolic acid (I), one of antibiotics first isolated from *Asperigillus fumigatus* by Waksman *et al.*,[4] has a growth-retarding effect on Gram-positive bacteria. Later, Okuda *et al.*[5] found that *Cephalosporium caerulenes* produced it in a much better yield than did *A. fumigatus*, and that this compound had a unique skeleton (protostane type)[6] of tetracyclic triterpenoid, in which the B-ring had a boat form. That is, this compound has a *trans*-syn-*trans* juncture in the A/B/C rings, unlike the *trans*-anti-*trans* juncture in usual steroids and triterpenoids. A revised absolute configuration has recently been presented based on extensive stereochemical and NMR studies of helvolic acid and its relatives.[7] In the course of this investigation, Inoue, Machida, and Okuda[8] have found that 6-oxohelvolic acid (IIa) or 1,2,24,25-tetrahydro-6-oxohelvolic acid (IIb) is very sensitive to oxygen in an alkaline solution, turning red or deep green respectively, gradually losing its color, and ultimately oxidizing into the anhydride (IIIa or b). These results, indicating a radical mechanism of the oxidation process, have prompted us to search for the presence of a relatively stable intermediate radical by making ESR measurements.

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1) L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, (1959); J. R. Hanson, "Introduction to Steroid Chemistry," Pergamon Press, London, (1967).

2) H. M. McConnell and B. G. McFarland, "Physics and Chemistry of Spin Labels," *Quart. Rev. Biophys.*, **3**, I (1970), pp. 91—136; O. H. Griffith and A. S. Waggoner, *Accounts Chem. Res.*, **2**, 17 (1969).

3) E. R. Talaty and G. A. Russell, *J. Amer. Chem. Soc.*, **87**, 4867 (1965); *J. Org. Chem.*, **31**, 3455 (1966).

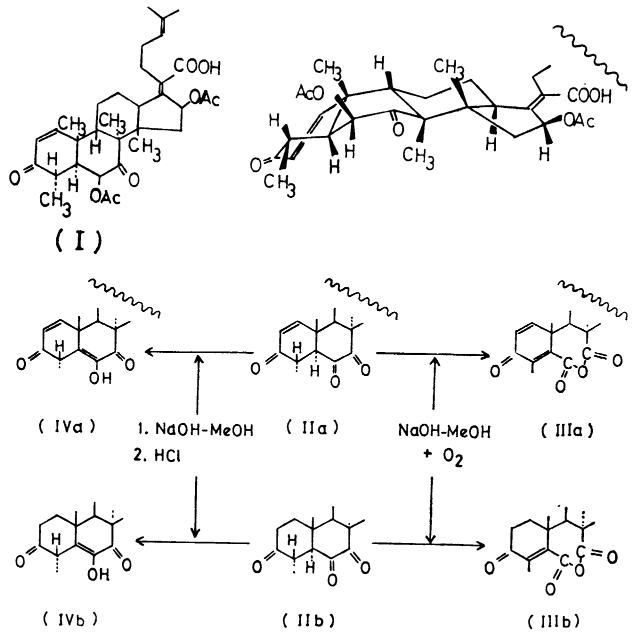
4) S. A. Waksman, E. S. Horning, and E. L. Spencer, *J. Bacteriology*, **45**, 233 (1943).

5) S. Okuda, S. Iwasaki, M. I. Sair, Y. Machida, A. Inoue, and K. Tsuda, *Tetrahedron Lett.*, **1967**, 2295.

6) As this skeleton is considered to be that of the prototype of sterol, the name protostane was proposed for the skeleton. T. Hattori, H. Igarashi, S. Iwasaki, and S. Okuda, *Tetrahedron Lett.*, **1969**, 1023.

7) S. Iwasaki, M. I. Sair, H. Igarashi, and S. Okuda, *Chem. Commun.*, **1970**, 1119.

8) To be published in *Chem. Pharm. Bull.* (Tokyo).



In this report, we will present some ESR results on the conformations of the triketone radicals—in other words, on that of helvolic acid.

Experimental

Materials. The triketones, IIa and IIb, were synthesized from helvolic acid in the laboratory of professor Okuda. The other reagents used were all of a G. R. grade.

Methods. ESR measurements were carried out at room temperature using a JEOL P-10 apparatus (Japan Electron Optics Laboratory Co., Ltd.) at the X-band and 100 KHz field modulation. The field calibration was made with reference to a standard sample of Mn^{2+}/MgO and a solution of Fremy's salt.

For the detection of an intermediate radical by the ESR method, an ESR sample tube with two compartments and a capillary tube was used in order to degas, introduce oxygen, mix the two solutions, and measure the ESR spectrum of the solution at room temperature.

Since an alkaline solution of the triketone is unstable toward oxygen, two separate solutions, one a neutral methanol solution of triketone and the other methanol containing KOH, were degassed thoroughly in each compartment attached to the sample tube by repeating the freeze-degas-thaw cycle; then the two solutions were mixed in one of the compartments at room temperature, followed by the introduction of an appropriate amount of oxygen. For the ESR measurements, a small amount of the colored solution was transferred into the capillary part of the sample tube, which was then inserted into the effective part of the microwave cavity.

The oxygen absorption kinetics were studied with a Warburg manometer.

Results and Discussion

It was confirmed that the two triketones, IIa and IIb, were very unstable toward oxygen in alkaline solutions, and were oxidized into the corresponding anhydrides, IIIa and IIIb. The molecular structures of the anhydrides were explained by the results of the UV, IR, and NMR measurements.[8] After a methanol solution of the triketone had been flushed with nitrogen and then made alkaline with 1 N NaOH, the color of the solution instantaneously changed from yellow to deep green or red in the case of IIb or IIa respectively. On the neutralization of this solution with HCl, the color of the solution returned again to yellow; enolized IIa (IVa) or its tetrahydro-derivative (IVb) was thus obtained in the yield of 100%.

It was found by Warburg's manometry that the amount of oxygen necessary to oxidize the triketone to its anhydride was one mole for one mole of triketone.

ESR experiments were carried out at room temperature, changing the concentration of the triketone in the range of 10^{-1}—10^{-3}M, and the concentration of alkali in the range of 1—1/100N.

According to the concentration of hydroxide relative to that of the triketone, various types of ESR spectra, A, B, C, D, and E (E′), with well-resolved hf structures and the *g*-factor of 2.004_3 were observed as is shown in Fig. 1. These spectra were fairly stable provided the amount of oxygen was not in excess, and they persisted even after one day.

With an increase in the hydroxide concentration relative to that of triketone, the ESR spectra were observed in the order of A, B, C, D, and E spectra for tetrahydro-triketone. For example, the mixed solution of 0.3 ml of 10^{-1}M tetrahydro-triketone in acetone, 0.05 ml of 2N NaOH, and 0.2 ml H_2O exhibited the D spectrum. After the observation of this spectrum, an appropriate amount of water was added, little by little, into the sample solution, and then the spectral type changed successively from D to C, to B, and finally to A. On the other hand, in the case of Δ^1-triketone, only the E′ spectrum was observed in a low hydroxide concentration. A distinct difference between the E and E′ spectra is that the latter has a well-resolved quartet hf structure in addition to that of the E spectrum.

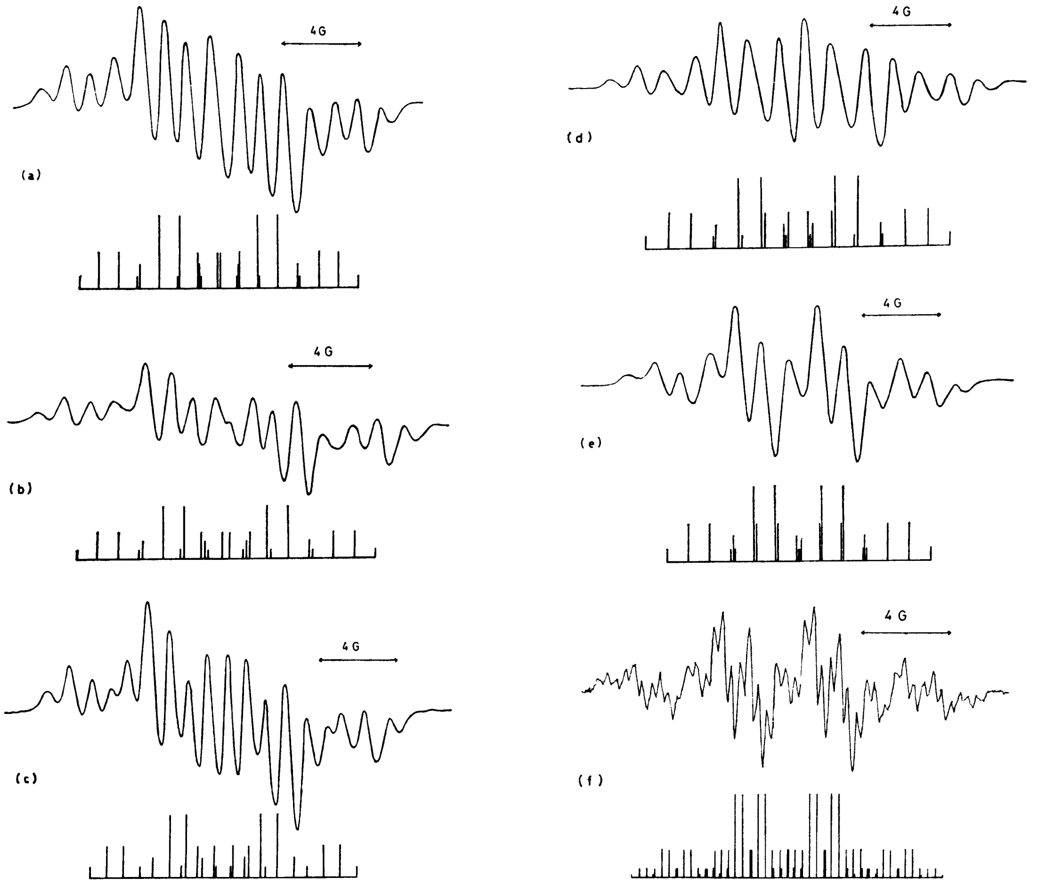
TABLE 1. HF SPLITTING CONSTANTS OF TRIKETONE RADICALS (in gauss)

Signal	No. of Lines	a_1(1H)[a]	a_2(2H)[a]	a_3(3H)[a]	a_4(3H)[a]
A	15	6.1	3.7	1.2	—
B	16	6.0	3.8	1.2	—
C	18	5.8	4.0	1.1	—
D	14	5.5	4.0	1.3	—
E	13	4.1	4.0	1.3	—
E′[b]	43	4.0	4.0	1.2	0.4

a) Numbers of equivalent protons.
b) Observed only for Δ^1-triketone.

All the spectra were analyzed; the respective stick diagrams are shown below the spectra in Fig.1. The hyperfine constants of each spectrum are tabulated in Table 1. Some of the computer-simulated spectra are also shown in Fig. 2.

It is obvious that these hf structures are attributable to certain protons in these radical structures, since the proton is the only nuclear species to show the hf structure. As can easily be seen from the splitting constants in Table 1, the finding that the observed spectral patterns changed in a delicate manner with an increase



(a) A spectrum:
5 mg tetrahydro-triketone + 0.4 ml A solution
A solution { acetone 30 ml; 2N NaOH in H_2O 5 ml; H_2O 20 ml

(b) B spectrum:
5 mg tetrahydro-triketone + 0.4 ml B solution
B solution { acetone 30 ml; 2N NaOH in H_2O 5 ml; H_2O 13 ml

(c) C spectrum:
5 ml tetrahydro-triketone + 0.4 ml C solution
C solution { acetone 30 ml; 2N NaOH in H_2O 5 ml; H_2O 6 ml

(d) D spectrum:
5 mg tetrahydro-triketone + 0.4 ml D solution
D solution { acetone 30 ml; 2N NaOH in H_2O 5 ml; H_2O 0 ml

(e) E spectrum:
0.3 ml of 10^{-2}M tetrahydro-triketone in 99% methanol + 0.3 ml of 0.3N KOH in 99% methanol

(f) E′ spectrum:
0.3 ml of 10^{-2}M Δ^1-triketone in 99% methanol + 0.3 ml of 0.03—0.3N KOH in 99% methanol

All the spectra were recorded at room temperature under the following operation condition; microwave power 3 mW, modulation width 0.05—0.3 G.

Fig. 1. ESR spectra of the triketone radical.

in the hydroxide concentration is due to a gradual decrease in the largest hf constant, a_1, down to the value of the second hf constant, a_2, while the third hf constant, a_3, remains nearly constant throughout, from the A spectrum to the E spectrum. On the other hand, in the E′ spectrum of the Δ^1-triketone radical, the hf splitting constants, a_1 and a_2, are already equal in a rather low concentration of hydroxide ions compared with the case of tetrahydro-triketone, and it has the smallest quartet hf splitting, $a_4=0.4$ G.

These spectral patterns were observed not only with the oxidation by molecular oxygen, but also with that by H_2O_2 or $Fe(CN)_6^{3-}$, and no change in the spectral patterns was observed even in the H_2O (D_2O), MeOH (MeOD), acetone, and DMSO solvents. From these results, it can be considered that the radical may be an ion-type radical produced by the one-electron oxidation of the triketones.

It is reasonable to consider that the radical has a structure spin-localized in the B-ring rather than in the A-ring, because essentially similar spectral patterns, E and E′, were observed with both triketones. Accordingly, the protons at the 1- and 2-positions are not responsible for the observed hf structure.

The proton at the 5-position is so labile that it is easily detached by enolization in an alkaline solution, as is confirmed by the finding that IVa or IVb is obtained in a full yield by alkalization and subsequent neutralization in the absence of oxygen. With regard to this nature of the 5α-H and the finding that no apparent change in the spectral pattern was observed in a CH_3OD or D_2O containing solvent, it can rea-

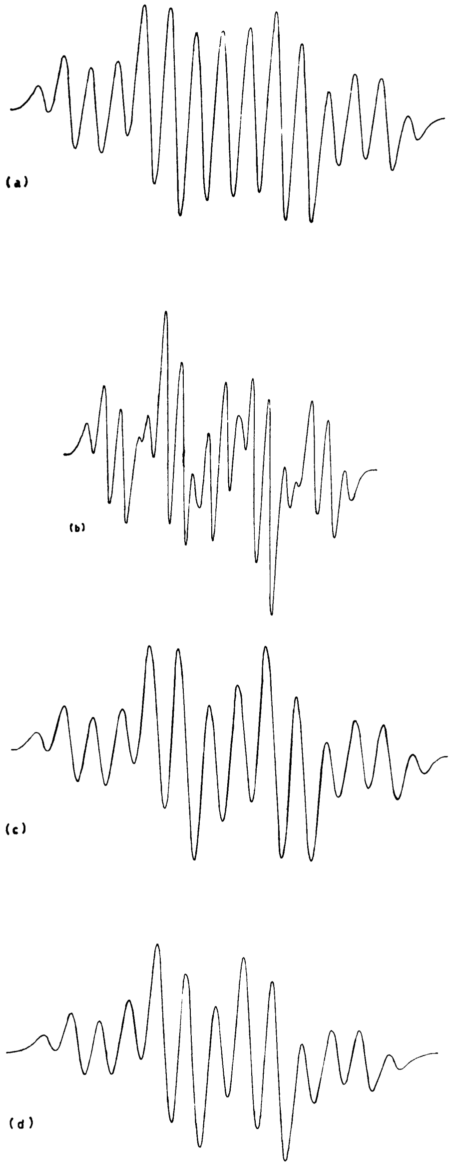


Fig. 2. Computer-simulated spectra of the triketone radical.
(a): A spectrum, (b): C spectrum, (c): D spectrum,
(d): E spectrum

sonably be concluded that this proton does not contribute to the hf structure either.

At first sight, it can easily be seen in Table 1 that each hf splitting constant makes two or three pairs of three equivalent protons, at least in the E and E′ spectra. This probably indicates that each hf splitting is due to a certain methyl group in the A- or B-ring. Let us consider which methyl group causes the corresponding hf splitting.

The enolization processes for Δ^1- or tetrahydrotriketone with an increase in the hydroxide concentration may be ascribed to the group shown in Fig. 3, in view of the degrees of stabilization by π-conjugation, the relative enolizabilities of protons at various positions, and the structures of the final oxidation products.

Canonical structures of reliable radical species produced by one-electron oxidation at each step are shown in the brackets. The spin density in these radicals is thought to be mainly localized at the carbon atom of the 5-position. The fact that the observed g-factor,

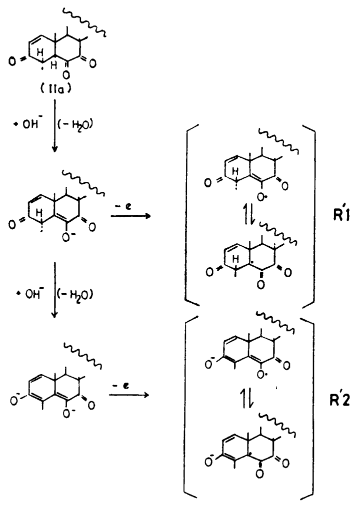


Fig. 3. Enolization processes for Δ^1-triketone and canonical structures of reliable radical species produced by one-electron oxidation.

$g=2.004_3$, is slightly larger than that of the free spin, $g_e=2.0023$, but much smaller than the empirical values of organic peroxy radicals,[9] $g_{av}=2.015$, is reasonably consistent with the envisaged radical structures.

It can easily be visualized, by means of the molecular models of the triketone radicals constructed on the basis of the confirmed *trans*-syn-*trans* juncture in

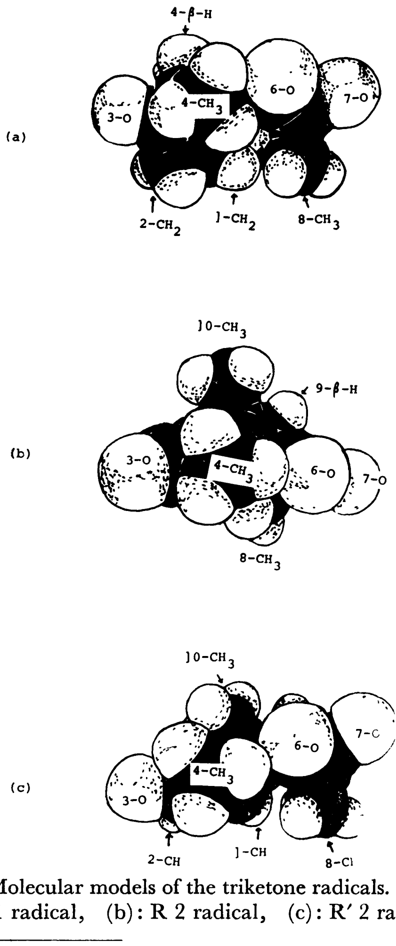


Fig. 4. Molecular models of the triketone radicals.
(a): R 1 radical, (b): R 2 radical, (c): R′ 2 radical

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9) M. Bersohn and J. R. Thomas, *J. Amer. Chem. Soc.*, **86**, 959 (1964); K. U. Ingold and J. R. Morton, *ibid.*, **86**, 3400 (1964).

the A/B/C rings of helvolic acid (or triketone), that the rotational motion of a certain methyl group in the triketone radicals is significantly affected with the progress of the step-by-step enolization of the 5α-H and 4β-H. Let us denote the radicals as R1 and R2 for the tetrahydro-triketone radicals, and as R′1 and R′2 for the Δ^1-triketone radicals which are produced by the one-electron oxidation at each enolization step. Molecular models of the R1, R2, and R′2 are shown in Figs. 4a, b, and c respectively.

First, the structure of the tetrahydro-triketone radical is discussed. In the structure of the R1 radical, the free rotation of the 4-CH_3 group is partly hindered by the steric hindrance due to the bulky oxygen atom at the 6-position; that of the 8-CH_3 is also hindered by the 1-CH_2 as is shown in Fig. 4a. On the other hand, it can be seen that the 10-CH_3 group, floating above the molecular plane, rotates freely notwithstanding a degree of enolization (this is also the case with the Δ^1-triketone radical). In the R2 radical, the 4-CH_3 is able to rotate freely, since the proton at the 4-position is removed, extending the π-conjugation and increasing the planarity of the A-ring with the progress of enolization. This situation is clearly shown in Fig. 4b. However, the steric hindrance upon the 8-CH_3 due to the 1-CH_2 can not be removed in spite of further enolization. This is comprehensible from the fact that the 8-position, being apart from the π-conjugation around the 3, 4, and 5-positions, is buried in the rigid structure of the molecules. It goes without saying that the 10-CH_3 is free to rotate.

Next, let us proceed to the structure of the Δ^1-triketone radical. In this radical, the planarity of the A-ring is large compared with that of the tetrahydro-triketone radical because of the presence of a double bond between the 1-C and 2-C atoms. Moreover, in accordance with the methine group, =CH–, at the 1-position in place of the –CH_2– group of the tetrahydro-triketone, there is no steric hindrance between the 8-CH_3 and the 1-CH before the enolization.

At the second step of the enolization, the free rotation of the 4-CH_3 group, which is partly hindered in the first step, becomes possible, as in the case of the tetrahydro-triketone radical.

It should be stressed here that a large spectral redshift in an alkaline solution was observed for Δ^1-triketone compared with tetrahydro-triketone, and that only one ESR spectrum was observed in the former. This result may indicate that the former is further subjected to the second step of the enolization under similar conditions, as can easily be understood from the structure of its ready π-conjugation.

It may thus be considered that the observed E′ spectrum corresponds to the R′2 radical, and that the smallest hf splitting constant of this radical can reasonably be assigned to the equivalent three protons of the 8-CH_3, since this hf splitting is observable only in the Δ^1-triketone radical. This is in accordance with the free rotation of this group; otherwise, such a small hf splitting might be obscured by the line-broadening due to hindered rotation, as is the case of the tetrahydro-triketone radical.

As to the third hf splitting constant, a_3=1.2 G, observed commonly throughout the spectra, A, B, C, D, E, and E′, it is safe to assign this to the 10-CH_3 group.

The above two assignments are reasonable in view of configuration of the spin-localization in the radicals.

The remaining assignments for the hf splitting constants of a_1 and a_2 are not so simple, because the hf splitting constant, a_1, gradually decreases from the 6.1 G of the A spectrum to the 4.1 G of the E spectrum, while, on the other hand, the a_2 value increases slightly from the 3.7 G of the A spectrum to the 4.0 G of the E spectrum. Since the 4β-H and 4α-CH_3 are close to the 5-position, where an unpaired electron is localized, and are common for both triketones, these protons can be expected to exhibit rather large hf splittings.

At the first enolization step, the rotation of the 4α-CH_3 group is so restricted that the protons in this group are not magnetically equivalent because of the steric hindrance and also partly because of a weak interaction, probably a hydrogen-bond-like one between the oxygen atom at the 6-position and two of the three protons of the 4α-CH_3 group; such a configuration is also supported by the similar interaction between the oxygen atom at the 3-position and the remaining proton of the methyl group. In such a configuration, one of the three protons of the methyl group is nearly equatorial, while the others are axial, to the plane of the A-ring. Therefore, the latter two protons may contribute equivalently to a hf splitting, while the former does not.

One can expect a larger hf splitting due to the 4β-H than that due to the 4α-CH_3, since the 4β-H is axial to the plane and is located closer to the 5-position of spin-localization. The variation in the hf splitting constant, a_1, during the first enolization process may be explained by a delicate change in the angle between the $2p_z$ axis of the 5-carbon atom and the C–H axis of the 4β-H.

At the final step of the enolization process, the 4β-H is removed, leaving the 4-CH_3 at the 4-position; correspondingly, in their ESR spectral patterns, the observed hf splitting becomes the one due to three equivalent protons—that is, a_1 (one proton)$=4.1\ \text{G} \fallingdotseq a_2$ (two protons)$=4.0$ G. Thus, the three protons of the 4-CH_3 group can be attributed to the equivalent observed hf structure of the E or E′ spectrum.

The above discussions are entirely based on the *trans*-syn-*trans* juncture in the A/B/C rings of helvolic acid; thereby, the hf splittings of the intermediate radicals are all reasonably explained. In other words, this result supports the conformation of helvolic acid, which has also been confirmed by other methods. The possibility of the epimerization of the A/B ring juncture during the oxidation reaction can reasonably be rejected on the basis of an analysis of the ESR spectra of the radicals; that is, such a conformational change obviously is not consistent with the observed hf splittings.

As has been described above, in the oxidation process of tetrahydro-triketone, the presence of an intermediate radical, R1 or R2, depending on the hydroxide

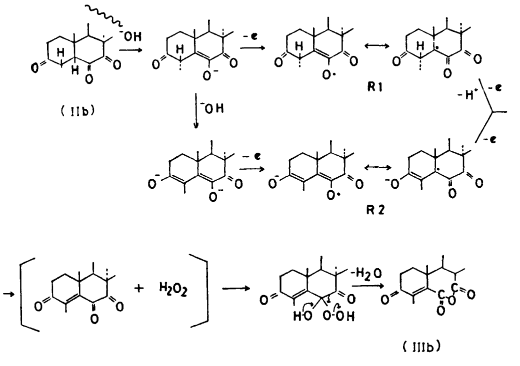

Fig. 5. The oxidation mechanism of the triketone to the anhydride.

concentration, has been confirmed by the ESR measurements, while, in the case of Δ^1-triketone, only the R′2 radical has been detected even in a rather low bydroxide concentration range. Therefore, the oxidation mechanism of the triketone to the corresponding anhydride *via* an intermediate radical may be shown as Fig. 5. In the case of tetrahydro-triketone, the reaction proceeds: IIb→R1→IIIb or IIb→R2→IIIb, depending on the hydroxide concentration. On the other hand, for Δ^1-triketone, only the way of IIa→R′2→IIIa is available independent of the hydroxide concentration, as can easily be understood from the above discussion.

Since the intermediate product, Int., has not been isolated in the present work, the rate-determining step is considered to be the one of its formation. The oxidation of the intermediate, thereafter, proceeds rapidly by the reaction with H_2O_2, which is formed in the preceding oxidation processes:

$$\left.\begin{array}{l}\text{the first one-electron oxidation step: } O_2+e=O_2^- \\ \text{the second one-electron oxidation step: } O_2+e=O_2^-\end{array}\right\}$$

$$\xrightarrow{+2H^+} 2HO_2 \longrightarrow H_2O_2 + O_2$$

The oxidation of ketones with H_2O_2, leading to the insertion of an oxygen atom into a steroid nucleus, is well known in steroid chemistry.[10]

The above oxidation mechanism accords well with the experimental findings that one mole of oxygen was consumed in the oxidation of the triketone to the anhydride, and that a trace of hydrogen peroxide was detected in the final oxidation products.

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[10] C. Djerassi, "Steroid Reactions," Holden-Day, Inc., San Francisco, (1963), pp. 457—535.